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An evaluation of sorter induced cell stress (SICS) on peripheral blood mononuclear cells (PBMCs) after different sort conditions - Are your sorted cells getting SICS?

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ABSTRACT

Flow cytometry and fluorescence-activated cell sorting have become invaluable tools to analyze and isolate specific cell populations in a wide range of biomedical research and clinical applications. In countless approaches worldwide, scientists are using single cell analyses to better understand the significance and variation within different cellular populations, and fluorescence-activated cell sorting has become a major technique for cell isolation in both basic and clinical research. However, majority of available cell sorters are pressurized, droplet-based systems, which apply significant environmental pressure and shear stress to cells during sorting. Recently, the flow cytometry community has become increasingly aware about the potential negative effects this could have on sorted cells and the term "sorter induced cell stress" (SICS) has been proposed. However, up to date only a limited number of studies have investigated the effects of cell sorting on cell viability and function. Therefore, solid data on the effects of sheath pressure and nozzle size on survival and function of sorted cells are surprisingly rare. With this in mind, we sorted "$CD4^+$" T-cells and "live" cells from human peripheral blood mononuclear cells (PBMCs) at different sort conditions and analyzed their quality before and after sorting in a series of assays. Here we present our findings in reference to cell viability and cell proliferation following sorting on different instruments (BD FACSAria III SORP and BD FACSJazz), utilizing different nozzle sizes (70 to 100 μm) and sheath pressure settings (20 to 70 psi). The results show no significant differences in cell viability and proliferation after the different tested sort conditions, but rather differences between individual experiments. These findings are evaluated and their potential significance in cell sorting experiments is discussed.

1. Introduction

A wide range of recent findings in biomedical research and clinical investigations are based on data obtained from specific cell populations purified by fluorescence activated cell sorting. Undoubtedly, cell sorting has widely become an accurate, quick and invaluable tool in many of these studies, representing a major technique for cell isolation in basic, clinical and translational research. However, most current conventional cell sorters are droplet producing systems, either "stream-in-air" or "cuvette" based. These instruments use pressurized sheath fluid, which passes through a narrow opening (nozzle) to generate droplets at high frequency with the help of an acoustic wave induced by an electric transducer. High sheath pressure – up to 70 psi (482.633 Pa) on commonly available instruments and high drop frequency allow for high speed cell sorting, easily reaching several thousands of events per second. Sorted cells can be used for various downstream applications, ranging from nucleic acid extractions for molecular investigations to various cell culture-based assays. Due to these principles, fluorescently activated cell sorting has become a fast, accurate, and reliable method to analyze and isolate a great number of different cell types and cell sorting facilities often represent basic infrastructure in biomedical research institutions around the globe.

The flow cytometry and cell sorting community have, however, become increasingly aware about potential negative effects that can potentially occur during sample preparation, cell sorting and collection. Several factors like abrupt and repeated temperature changes, antibody

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activation, high pressure, shear stress, high voltage electric fields or laser irradiation are present during the cell sorting workflow and can eventually impact sorted cells and data obtained from downstream applications (Fig. 1). It is because of such concerns that the term "sorter induced cell stress" (SICS) has been proposed by Lopez and Hulspas (Lopez and Hulspas, 2020) in the field of cell sorting to ensure rigor and reproducibility of flow cytometric data. However, such effects and possible consequences are often passed on as observations from one sorter lab to the other, but published data is surprisingly rare to find. There is certainly need for robust data on potential effects of fluorescently activated cell sorting on cell viability and function, even though a limited number of published data on specific aspects of this topic are available by now (Andrä et al., 2020; Beliakova-Bethell et al., 2014; Binek et al., 2018; Richardson et al., 2015; Sinha et al., 2011).

With this lack of published data in mind, we sorted $CD4^+$ T cells and "live" cells from human peripheral blood mononuclear cells (PBMCs) at different sort conditions using two different cell sorters (BD FACSAria III SORP and BD FACSJazz) in this study. 100-µm nozzles (BD FACSAria and BD FACSJazz) and 70-µm nozzle (BD FACSAria) were tested with corresponding sheath pressure settings of 20, 27 and 70 psi (137.895, 186.158 and 482.633 Pa respectively). Viability of sorted $CD4^+$ and "live" cells was monitored before and after each sort to evaluate a potential impact on the overall quality and survival of cells in dependence of nozzle size and sheath pressure. Unsorted cells were included in all our assays as a control. In addition, we looked at cell proliferation upon stimulation with anti-CD3 and anti-CD28 antibodies for up to 7 days after each sort, using CFSE-based cell proliferation assay, as an indicator of cellular function.

2. Materials & methods

2.1. Samples

Buffy coat samples collected from healthy individuals ($n = 3$) were obtained from the Blood Donor Center at Hamad Medical Corporation Doha, Qatar. PBMC were isolated by standard density-gradient centrifugation using Histopaque-1077 (Sigma-Aldrich, Irvine, U.K.). PBMC were frozen in cryovials at a density of 5 million cells per 1 ml freezing

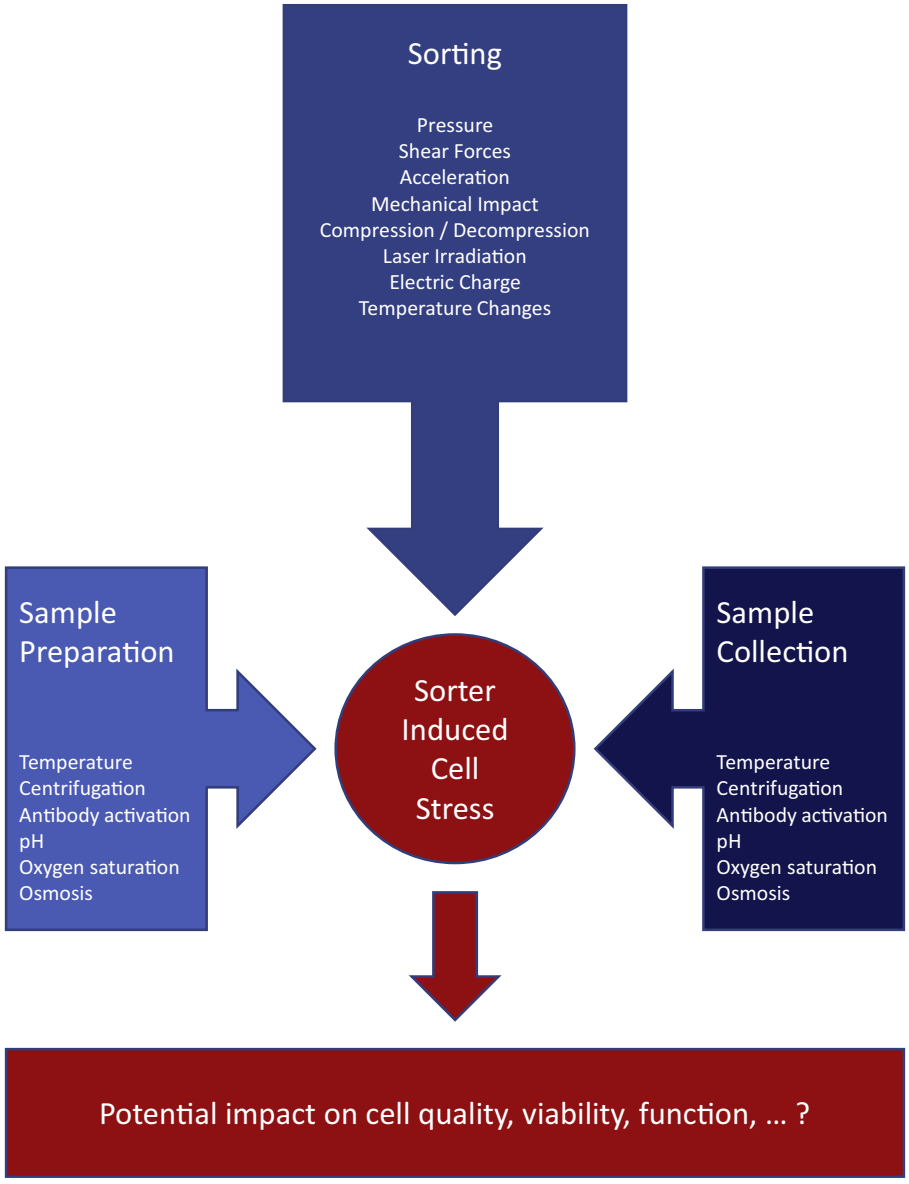


Fig. 1. Several factors can lead to sorter induced cell stress (SICS) in Fluorescence-Activated Cell Sorting (FACS). Schematic representation of possible factors that can induce SICS on droplet-based FACS during cell preparation, sorting and cell collection.

media [50% fetal bovine serum (FBS), 40% RPMI-1640 media and 10% dimethylsulfoxide (DMSO)] to be used in batches for subsequent analyses. This study was performed under an ethical approval from Qatar Biomedical Research Institute, Doha, Qatar (Protocol no. 2017–006). All experiments were performed in accordance with relevant guidelines and regulations.

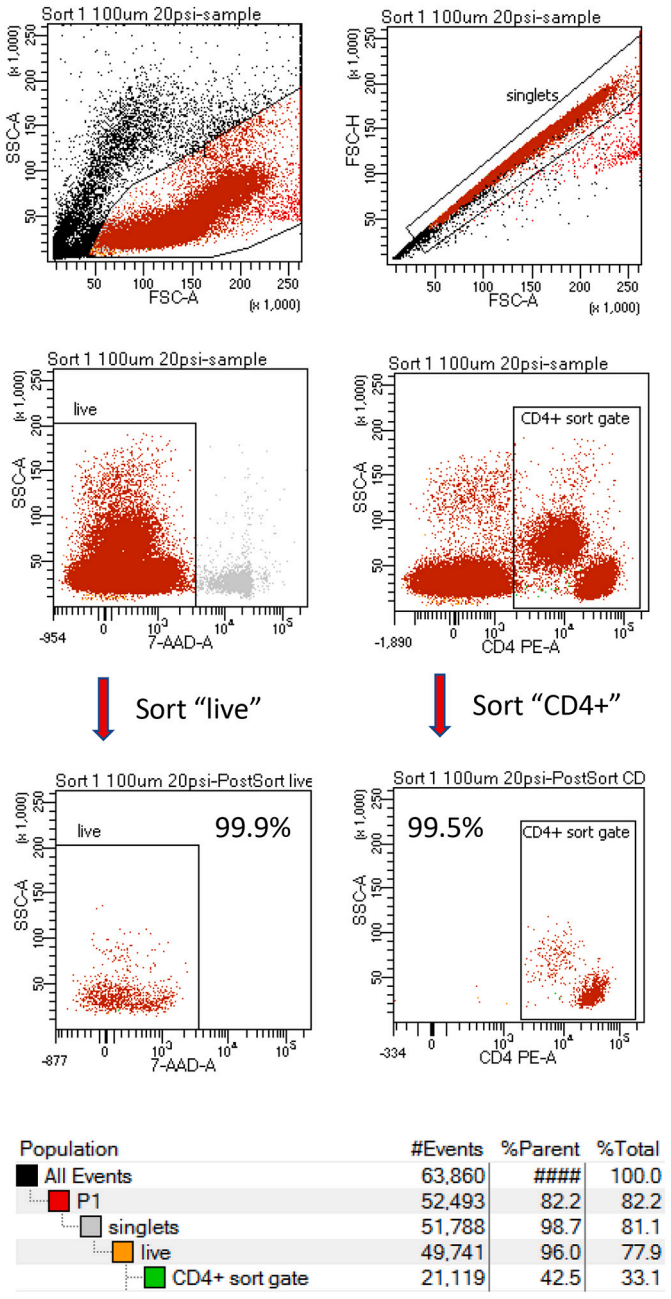
2.2. Flow Cytometry and cell sorting

Frozen PBMC were thawed and stained with anti–CD4-PE (clone RPA-T4; BD Biosciences) and 7-aminoactinomycin D (7-AAD, BioLegend) after blocking with FcR Blocking Reagent (Miltenyi Biotec). After staining, cells were filtered (25 µm Filcon sterile cup-type Filter, BD Biosciences) and 7 to 10 million cells were diluted in 1.5 ml complete medium (RPMI-1640 supplemented with 2 mM L-glutamine, 10% FCS and 1% penicillin/streptomycin) for immediate sorting. $CD4^+$ T cells and $CD4^{Intermediate}$ monocytes were sorted as combined $CD4^+$ cells in parallel using the BD FACSARIA III SORP and BD FACSJazz Cell Sorters (BD Biosciences). In addition, all live cells (7-AAD negative) were sorted as controls for each sort. Three sort conditions were tested in three replicates each (BD FACSAria: 100 µm nozzle / 20 psi sheath pressure and 70 µm nozzle / 70 psi sheath pressure, BD FACSJazz: 100 µm nozzle / 27 psi sheath pressure). BD FACSFlow sheath fluid (BD Biosciences) was used to operate all sorters and manufacturer's standard startup-, cleaning- and QC procedures were applied before each sort. 405 nm (45 mW), 488 nm (95 mW), 561 nm (45 mW) and 641 nm (55 mW) lasers were active during sorts on the BD FACSAria III SORP, and 405 nm (50 mW), 488 nm (80 mW) and 640 nm (50 mW) standard lasers were active during sorts on the BD FACSJazz. Standard optical filters and mirrors configurations were used for detection of scatter and fluorescence signals. All sorts were conducted at room temperature (sample) and sorted cells were collected in cooled (10° Celsius on BD FACSAria SORP, ice-covered on the BD FACSJazz) 5 ml round-bottom polypropylene tubes (Corning) in 500 µl RPMI-1640 medium supplemented with 10% FCS. Only single cells were selected for sorting through gating and dead cells were excluded by 7-AAD (Fig. 2). Immediately after the sort, sorted samples and an unsorted control sample were centrifuged at 1.500 rpm and 4° Celsius for 5 min, supernatant was discharged and cells were diluted and kept in 2 ml complete medium per million cells overnight (37° Celsius, 5% CO_2).

3. Cell viability and CFSE proliferation

In addition to cell viability analyses by 7AAD, cell proliferation was measured by Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE)-based proliferation assays, as described previously (Lyons and Parish, 1994; Toor et al., 2019). We investigated proliferation of sorted "$CD4^+$" and "live" populations after each sort for up to 7 days. Un-sorted cells were treated the same way as sorted samples and included in all assays as controls. We started all our proliferation assays 24 h after each sort to allow the sorted cells a standardized recovery and resting period prior to each proliferation assay.

For proliferation assays, 1×10^5 sorted $7AAD^-CD4^+$, $7AAD^-$ cells, and un-sorted PBMCs were cultured in duplicates, after labelling with CFSE. Cells were stimulated with soluble anti-CD3 (clone OKT3, eBioscience) / CD28 (clone CD28.2, eBioscience) antibodies (2 µg/ml) in 96-well round-bottom tissue culture-treated plates. CFSE staining was assessed for each assay at the beginning of each stimulation (day 0) and all CFSE positive cells were gated on as starting point for non-proliferating cells. Proliferation, viability and scatter were then evaluated by collecting cells for flow cytometry after 3 to 7 days on a BD FACSAria III SORP cell sorter. Laser power, PMT-voltage settings and gating were kept constant for the duration of the entire experiments (days 3 to 7) and percentage of proliferating cells was analyzed on the same instrument (BD FACSAria III SORP) by calculating the portion of cells with loss in CFSE signal intensity as compared to the CFSE gate set at day 0.



Population	#Events	%Parent	%Total
All Events	63,860	####	100.0
P1	52,493	82.2	82.2
singlets	51,788	98.7	81.1
live	49,741	96.0	77.9
CD4+ sort gate	21,119	42.5	33.1

Fig. 2. Sorting strategy of live and $CD4^+$ T cells. Representative flow cytometric plots show the sorting/gating strategy of $CD4^+$ (PE) and "live" (7AAD) populations from PBMC (BD FACSAria III SORP, 100 µm nozzle / 20 psi sheath pressure).

4. Results

4.1. Cell sorting using different sort conditions

We sorted "$CD4^+$" and "live" cells from frozen PBMCs at three different conditions and on two different cell sorters (100 µm / 20 psi and 70 µm / 70 psi on the BD FACSAria III SORP and 100 µm / 27 psi on the BD FACSJazz). All tested conditions represent standard sort setups for these instruments. The purity for each cell type after sorting was high for all sorts (Fig. 2). Average event rates during sorts were kept at 3000 events per second, achieved by adjusting flow rate between 1 and 2 on the BD FACSAria, and sample offset between 1.5 and 1.7 psi on the BD

FACSJazz. These settings combined with a standard "Purity" sort mode on both instruments resulted in sort efficiency rates between 90 and 98% and yield between 850.000 and 1.5 million purified cells for all sorts. Independent of instrument and sort condition, each sort was completed after around 30 min. Overall, all tested sort conditions were similar in terms of sorting time, sort efficiency, achieved purity and yield of sorted cells.

4.2. Effects of cell sorting on cell viability

Prior to each sorting experiment, PBMC viability was tested by 7AAD and only samples with more than 90% viable cells of interest (pre-gated on FSC/SSC) were used for the experiments (Fig. 2). As both sorted populations "$CD4^+$" and "live" applied a sort gate on 7AAD negative cells (representing viable cells), the viability of purified cells immediately after each sort was close to 100% as confirmed by 7AAD (data not shown). After each sort cells were kept in complete medium (37° Celsius, 5% CO_2) overnight before CFSE staining and proliferation assays were started (day 0). Cell viability at day 0 was assessed by 7AAD immediately after CFSE labelling and before culturing.

Mean viability of sorted $CD4^+$ cells was between 94 and 95% on day 0 for all tested sort conditions, following resting (Fig. 3A). No difference in viability was seen between the different nozzle sizes / sheath pressure settings at this time. However, a relatively high standard deviation reflects a considerable variation between experiments, rather than between sort conditions. After 3 days of proliferation in cell culture, mean cell viability dropped slightly to around 90% (88–92%), showing more variation between experiments than between different sort conditions. There were no significant differences in mean viability between sorted $CD4^+$ cells and un-sorted cells at day 0 and day 3 (Fig. 3A), but some experiment specific differences were observed (e.g. lower viability of un-sorted cells after 3 and 4 days in Fig. 3B). Mean viability of sorted "live" cells was similar to the data shown for $CD4^+$ cells in all our experiments (data not shown). Overall, viability of sorted cells was high for all tested sort conditions and up to 7 days cell culture (Fig. 3).

4.3. Effects of cell sorting on cell proliferation

As indicated by high viability, both $CD4^+$ and "live" sorted cells were generally in good condition and proliferated well after sorting, independent from the applied sort conditions (Figs. 4 and 5). After three days stimulation with anti-CD3 and anti-CD28 antibodies an average of around 50% (42–62%) of sorted $CD4^+$ and "live" cells were proliferating after all three tested sort conditions. Importantly, un-sorted cells showed equal proliferation as the sorted populations after 3 days (Fig. 4A). The percentage of proliferating cells increased to around 60% (52–71%) in $CD4^+$ and "live" sorted cells after 4 days stimulation, around 80% (66–85%) after 5 days stimulation and around 90% (85–92%) after 7 days of stimulation. Moreover, un-sorted control cells displayed similar percent of proliferating cells as $CD4^+$ and "live" sorted cells for each day (Fig. 4A). In line with our results from the cell viability data, we did not find any significant differences in proliferation in the different sort conditions, but rather high variations from experiment to experiment (see high SD in Fig. 4A). This was confirmed by looking at the proliferation data from each individual experiment. The percentage of proliferating cells of sorted populations were not different between the different sort conditions within one experiment, but different between different experiments (Fig. 4B).

4.4. Effects of cell sorting on phenotypical characteristics

To investigate the phenotypical characteristics of proliferating cells in more detail and over the course of stimulation, we investigated the scatter properties, CD4 expression and CFSE profile on CD4+ sorted cells and unsorted cells on day 0 and after 3, 4 and 5 days of stimulation. As indicated by the percentage of proliferating cells, the FSC/SSC profiles of

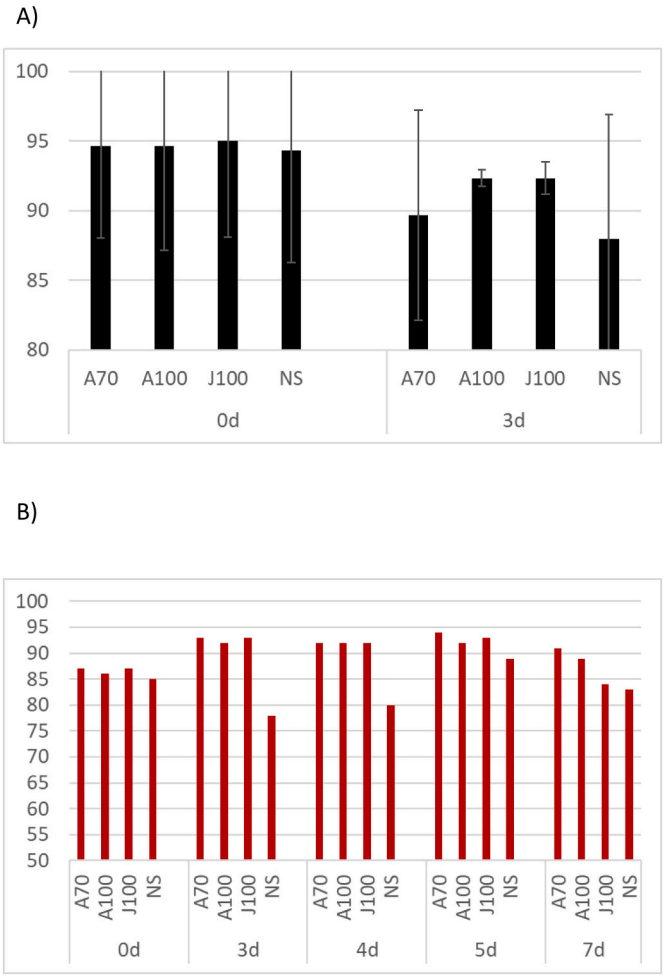


Fig. 3. Cell viability (% 7AAD negative cells) after sorting in different conditions. Live/$CD4^+$ T cell populations were sorted using different conditions including, A100: BD FACSAria 100 µm nozzle / 20 psi sheath pressure, A70: BD FACSAria 70 µm nozzle / 70 psi sheath pressure, J100: BD FACSJazz 100 µm nozzle / 27 psi sheath pressure (NS: un-sorted. 0d: immediately after sort, 3d: 3 days after sort, 4d: 4 days after sort, 5d: 5 days after sort). Bar plots show the mean viability (% 7AAD negative cells) +/− SD of $CD4^+$ sorted cells and un-sorted cells (NS) from three independent experiments (A). Bar plots show the viability (% 7AAD negative cells) of $CD4^+$ sorted cells from single experiment (B). Note that the last columns for each day are un-sorted cells (NS) for comparison.

both $CD4^+$ sorted and un-sorted cells displayed normal and apparently healthy cell characteristics with proliferating cells moving up on both FSC and SSC axes as they proliferated (Fig. 5A). Other indication for an unimpaired proliferation pattern in $CD4^+$ sorted and un-sorted cells was displayed by the progression of CD4 together with CFSE over time (Fig. 5B). As expected, proliferating cells gradually lost CD4 positivity as they proliferated, whereas the non-proliferating cells remained positive in terms of CD4 expression (blue populations in Fig. 5B). The pattern of the un-sorted cells revealed that both $CD4^+$ and $CD4^-$ cells proliferated normally, and no differences in proliferation patterns were observed for the $CD4^+$ populations in the differently sorted versus the same population present in the un-sorted sample (Fig. 5B).

5. Discussion

Numerous flow cytometry applications are common and essential tools in modern biomedical and biotechnology research, translational studies and clinical diagnosis (Macey, 2007; Mattanovich and Borth,

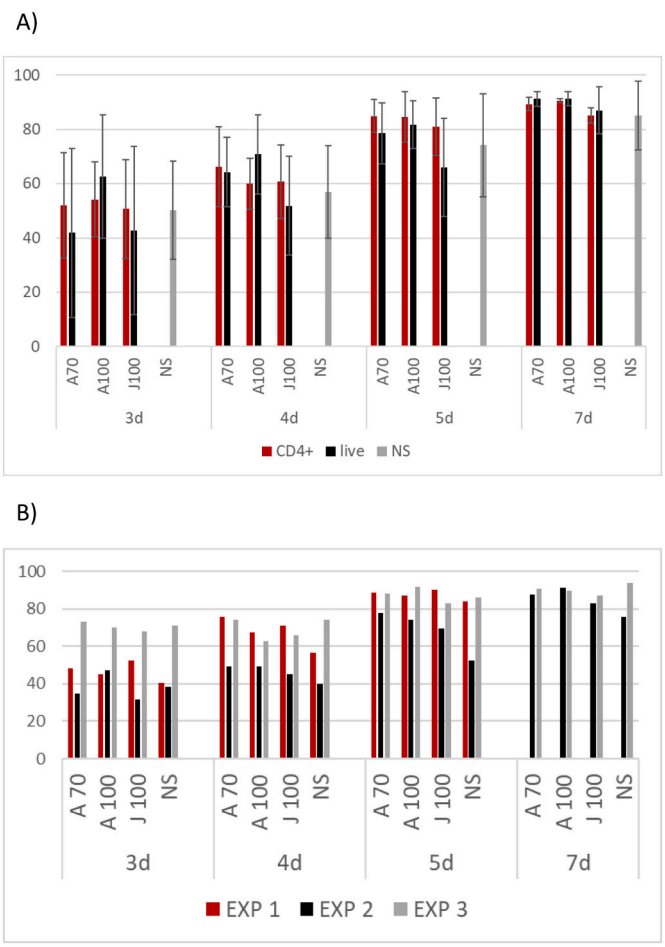


Fig. 4. Post-sorting cell proliferation analyses. Cell proliferation determined by CFSE staining after sorting $CD4^+$ and "live" populations at different conditions (A100: BD FACSAria 100 µm nozzle / 20 psi sheath pressure, A70: BD FACSAria 70 µm nozzle / 70 psi sheath pressure, J100: BD FACSJazz 100 µm nozzle / 27 psi sheath pressure, NS: un-sorted. 3d: 3 days after sort, 4d: 4 days after sort, 5d: 5 days after sort, 7d: 7 days after sort) and 3 to 7 days stimulation with soluble anti-CD3 and anti-CD28 antibodies. Bar plots show the mean % of proliferating cells (CFSE negative, +/− SD) for "$CD4^+$", "live" and un-sorted cells (A). Bar plots show the % of proliferating "$CD4^+$" sorted and un-sorted cells from three independent experiments (B).

2006; Virgo and Gibbs, 2012; Brown and Wittwer, 2000; Sack et al., 2008). Common analyses include immunophenotyping, detection and measurement of protein expression and post-translational modifications, cell cycle- and apoptosis analyses, and determination of the transfection efficiency of expressing a gene by using a marker, such as GFP. Many of such analyses rely on additional functional downstream studies, which require specific, physically isolated, and ideally unaltered cell populations obtained by fluorescently activated cell sorting (FACS) (Mattanovich and Borth, 2006; Ibrahim and Van Den Engh, n.d.; Davies, 2007).

In recent years, cell sorting has become a standard technique in numerous labs around the world and concerns about potential negative effects that can occur during sample preparation, sorting and cell collection are increasingly risen. Available studies indicate that cell sorting can alter the expression of certain genes in isolated cell populations from mouse mammary glands (Richardson et al., 2015) and in sorted human leukocyte subsets (Beliakova-Bethell et al., 2014). On a poster presented at the 2014 Association of Biomedical Resource Facilities (ABRF) Jurkat cells were reported to suffer loss of cellular integrity and displayed altered gene expression a few hours after having been sorted at intentionally harsh conditions. In the same study, the authors state that sorted cells could recover from the sort after a few hours and observed alterations in gene expression were substantially decreased 8 h after the sort (Seymour, 2014). Other studies report the alteration of cellular redox state on rat astrocytes (Llufrio et al., 2018) and changes of the metabolome in mouse macrophages (Binek et al., 2018) after sorting. A recent study revealed the impact of cell sorting on T cell functionality by p38 MAPK activation (Andrä et al., 2020). Overall, some specific impact on certain cells can be observed after sorting, but conclusive data on long-term cell viability and function after sorting is missing, as cells were often collected for downstream analysis immediately after the sort in these studies. With the current study, we aim to contribute to a better understanding of possible long-term effects of cell sorting on the viability and function of different sub-populations of human PBMCs after undergoing three different sort conditions on two different cell sorter models.

All three tested sort conditions using the BD FACSAria III SORP and the BD FACSJazz cell sorters resulted in pure, viable and functional sorted human PBMC cell populations, revealed by 7AAD viability tests and CFSE proliferation assays. No differences were observed between differently sorted and un-sorted control cells, indicating none or only minor negative impact of sorting on human PBMCs at the tested conditions. Sort speed, sort efficiency, sort yield and quality of sorted cells were comparable for all sorts and consistent between different sort conditions and instruments. We had all lasers except the UV laser active during all sorts to give a realistic idea for multicolor experiments. Thus, we are aware that our results only reflect possible influence of sheath pressure and nozzle sizes on the quality of sorted cell populations, but do not reflect any possible effects of cellular exposure to different laser light. Although not tested in this study, laser wavelength and laser power can have additional negative effects on cell viability and function, especially for certain sensitive cell types, and future experiments are needed to reveal such eventual effects.

Our results showed that sorting did not have any negative impact on cell viability under all tested sort conditions. High cell viability was consistent between different sort conditions and for a period of up to seven days after the sort. From these results we conclude that human PBMC can survive standard sort conditions perfectly well and neither short-term (immediately after the sort) nor long-term (several days after the sort) viability of sorted cells was impaired. Interestingly, in some of our experiments viability of unsorted control cells was even slightly lower than viability of sorted cells. This could be because we applied a "live" gate for all our sorts, thus selecting for viable cells and removing dying or dead cells from the sample. It seems likely that by doing so we might give the sorted cells a better "dead-cells free" environment and an advantage to survive as compared with their unsorted counterparts.

The results from our CFSE proliferation assays are in line with the viability data. Both $CD4^+$ and "live" sorted PBMC did proliferate well after sorting at each tested condition and up to seven days, indicating no negative impact of nozzle size and sheath pressure on the cell's ability to proliferate. Observed proliferation rates were within rates expected from literature data (Gett and Hodgkin, 1998; Li and Kurlander, 2010). As for the viability assays, we found higher variation in proliferation activity between different experiments as compared to different sort conditions. Although slightly higher than expected, such variations between experiments are not surprising and can be explained by accumulation of minor differences in the quality of original sample, PBMC isolation workflow, freezing/thawing procedure, staining protocols, sorting/cell collection, cell culture environment and antibody stimulation – even when following standardized protocols and workflows. In summary, our proliferation data suggest that experimental factors have a higher impact on PBMC viability and proliferation as compared to sorting them at the applied conditions.

We tested cell viability via 7AAD staining and cell function via CFSE proliferation on frozen human PBMC stained for CD4 (PE). These are both standard assays and are widely used in immunological flow cytometry. However, using only these two assays on one relatively

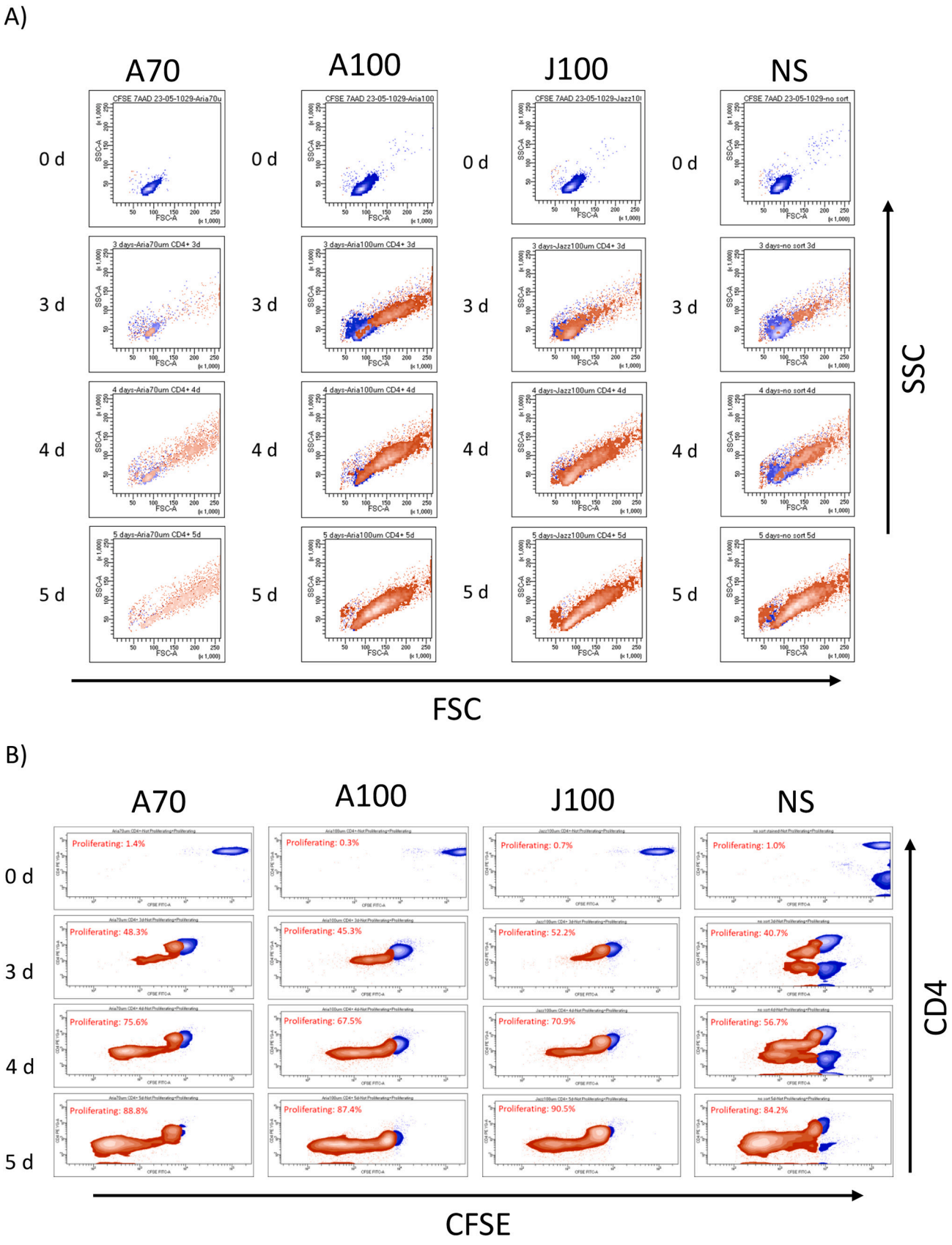


Fig. 5. Post-sorting cell proliferation analyses of $CD4^+$ T cells. Proliferation of "$CD4^+$" (PE) sorted cells with different sort conditions (A100: BD FACSAria 100 µm nozzle / 20 psi sheath pressure, A70: BD FACSAria 70 µm nozzle / 70 psi sheath pressure, J100: BD FACSJazz 100 µm nozzle / 27 psi sheath pressure, NS: un-sorted. 0d: start of proliferation assay, 3d: 3 days after sort, 4d: 4 days after sort, 5d: 5 days after sort) were determined by CFSE staining after 0, 3, 4 and 5 days stimulation with soluble anti-CD3 and anti-CD28 antibody. Flow cytometric plots show the forward versus side scatter (FSC vs. SSC) of cells (A) and progression of CFSE on $CD4^+$ T cells from $CD4^+$ sorted and un-sorted cells (B) at aforementioned sort conditions after 3 to 5 days of stimulation.

robust cell type may be a limitation. However, this gave us the possibility to test common and immunological relevant human cell populations sorted under different realistic conditions in a standardized way and in a decent timeframe. Furthermore, we found that the percentage of $CD4^+$ T cells did not differ before and after all tested sort conditions, and the percentage of $CD4^{dim}$ monocytes was only marginally decreased (from 17 to 18% before to 11–14% after sorting). Although this slight decrease could be the result of higher vulnerability to sorting, it could also be explained by loss of some monocytes for re-analysis after sorting due to the fact that they easily attach to the surface of the collection tubes during sorting. The combination of 7AAD and PE might appear as a suboptimal choice in terms of fluorescence spillover, but (Lopez and Hulspas, 2020) we wanted to use these two fluorochromes as commonly used by us and many other labs, (Andrä et al., 2020) neglectable spillover was observed in our experiments as we excited PE by 561 nm laser and 7AAD by 488 nm laser, and (Beliakova-Bethell et al., 2014) we only used 7AAD negative live cells for sorting and analysis, thus practically eliminating fluorescence spillover between PE and 7AAD. Furthermore, only stimulation with soluble anti-CD3 and anti-CD28 antibodies is reported here. Bead-based stimulation with the same antibodies was performed in parallel for all our experiments to investigate if the type of stimulation would have an influence on PBMC proliferation. However, both soluble and bead-bound antibody stimulation consistently revealed similar results, suggesting that the type of stimulation had no influence on the outcome of our proliferation assays. Overall, we are confident that our data is conclusive and reflects some of the most common sorting situations in immunological studies accurately. In conclusion, our data show negligible effects of sorter induced cell stress (SICS) on sorted PBMC populations at the tested sort conditions and parameters. However, further investigations and more complex functional assays together with data from genomics and proteomics are required to evaluate the effects of sorting on small, unique subpopulations of lymphocytes and larger, potentially more fragile cells.

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